Journal of Kerman University of Medical Sciences

JKMU

Journal of Kerman University of Medical Sciences, 2019; 26 (2): 110-119

Angiogenesis and Tumor-Associated Macrophages in Different Grades of Oral Squamous Cell Carcinoma, Verrucous Carcinoma and Epithelial Dysplasia via Immunohistochemical Assessment of Expression of CD34 and CD68 Markers

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Received: 9 February, 2019 Accepted: 11 Marc, 2019

ARTICLE INFO

Article type:
Original Article

Keywords:
Angiogenesis
Macrophage
Squamous Cell Carcinoma
CD34
CD68

Abstract

Background: Squamous cell carcinoma (SCC) is the most common malignancy of the oral cavity, which is highly invasive. Verrucous carcinoma (VC) is the low-grade form of SCC. Epithelial dysplasia (ED) also has a malignant potential. This immunohistochemical (IHC) study aimed to assess angiogenesis and the presence of tumor-associated macrophages (TAMs) in SCC, VC and ED to determine the role of these factors in the progression of dysplastic lesions to neoplasia.

Methods: Two 4 µ-thick sections were made of 43 paraffin blocks (14 SCC, 14 VC and 15 ED lesions confirmed by two pathologists) for IHC staining. The mean microvessel density (MVD) and the number of TAMs were determined by assessing the expression of CD34 and CD68, respectively in each group of lesions. Data were analyzed using the Fisher's Exact Test, Chi-square, Kruskal Wallis tests and one-way ANOVA with the aid of SPSS version 21.

Results: Expression of CD34 in ED was higher than that in VC and SCC (ED>VC>SCC). Expression of this marker in more severe forms of ED was higher than that in mild forms. This expression was lower in high-grade SCC in comparison to low-grade SCC. Expression of CD68 in SCC was slightly higher than that in VC and ED. Expression of this marker in severe ED was less than that in mild ED. Its expression in high-grade SCC was higher than that in low-grade SCC, but these differences were not significant for CD68. No significant association was noted between the expression of CD68 and CD34 in these lesions.

Conclusion: The increase in the number of TAMs in malignant oral epithelial lesions was related to the type of tumor and its histopathological grade, but no association was found between TAMs and MVD.

Citation: Saghafi SH, Zare Mahmoud Abadi R, Basir Shabestari S, Fazel SH, Shirinbak I. Angiogenesis and Tumor-Associated Macrophages in Different Grades of Oral Squamous Cell Carcinoma, Verrucous Carcinoma and Epithelial Dysplasia via Immunohistochemical Assessment of Expression of CD34 and CD68 Markers. *Journal of Kerman University of Medical Sciences*, 2019; 26 (2): 110-119.

Introduction

Oral Cancer (OC), the 6th most prevalent cancer, is a malignancy originated from the oral and pharyngeal mucosal tissues. SCC is the most common type of OC. Oral SCC often happens following the occurrence of oral Epithelial Dysplasia (ED) or oral Verrucous Carcinoma (VC) (1). Biopsy and subsequent immunohistochemical (IHC) analysis of the tissue by using specific antibodies against dysplastic cell markers is the most reliable method to detect dysplastic epithelium (2). Unresponsiveness to treatment may occur in some patients despite advanced technologies in the field of surgery and radiation therapy (3). Therefore, enhancement of the knowledge regarding relevant biomarkers and their impact on therapeutic responses may improve prognosis (4,5).

Angiogenesis refers to the formation of new blood vessels (6) and occurs as the result of an imbalance between the natural inhibitors and angiogenic stimulators (7). It seems that tumor angiogenesis in the initial preneoplastic phases is activated by the development of tumor and is regulated by a number of positive and negative mediators produced by the cancer cells and tumoral leukocytes (8). Endothelial cells and also stromal cells such as macrophages can be activated through pro-angiogenic molecules produced by transformed cells. Angiogenesis has an essential effect on tumor progression, tumor cell dissemination, local invasion, and metastasis by providing nutrients and growth factors (9). Vascular Endothelial Growth Factor (VEGF) is an important stimulant of angiogenesis secreted by the tumoral and normal cells, which affects the endothelial cells and plays an important role in tumor angiogenesis and also metastasis (9,10). Evidence has documented the role of Mean Microvessel Density (MVD) as a prognostic factor in solid tumors, and many studies have confirmed the correlation of VEGF and angiogenesis in tumors and their invasion potential; however, a few studies have refuted this correlation (6,9). Endothelial cells are identified by the use of endothelial markers with variable sensitivity and specificity depending on their stage of development, pathological and physiological status and the target organ (11). CD31, CD34, VEGF, factor VIII, von Willebrand factor and vimentin are among the endothelial markers used for the assessment of angiogenesis (11,12).

There is a growing interest to evaluate the relationship between inflammation and cancer (13,14). In neoplastic lesions, macrophages are found in extracellular matrix of the tumor known as the Tumor-Associated Macrophages (TAMs) (8). They are usually present in head and neck SCC (HNSCC) with variable degrees (4). It is stated that TAMs may have prognostic value in some neoplasms, because they act as important effectors, inducing a pro-angiogenic outcome during the 'angiogenic switch', and play a vital role in stimulating tumor associated angiogenesis (15,16). It is believed that TAMs induce angiogenesis by secreting angiogenesis-promoting factors such as tumor necrosis factor alpha (TNF-a), VEGF, angiogenin and urokinase. As a result, some studies suggest that in some cancers TAMs are associated with poor prognosis (12,15). Liss provided evidence that HNSCC tumoral cells absorb monocytes or TAMs and produce cytokines that stimulate the release of interleukin (IL) 1a, TNF-a and high levels of VEGF and IL-8. (15). TAMs express several macrophage-specific markers including CD68, CD23, and CD14 (17). CD68 is the most recognized marker (18).

It is unclear if TAMs and MVD could play a role as prognostic markers of oral cancers (19). Quantification of angiogenesis can serve as a surrogate marker in premalignant

lesions for the assessment of tumor development (20). Taking into account the gap of information on the association of MVD and TAMs in oral VC, ED and SCC (21) as well as the controversies in this field, our study aimed to assess the expression of CD34 and CD68 IHC markers to determine the presence of angiogenesis and TAMs. We also looked for their correlation in different grades of VC, SCC and ED in order to reveal any potential theoretical frameworks for using these markers in early detection and intervention.

Materials and Methods

This in vitro study was performed on 43 specimens including 14 specimens diagnosed with oral SCC (five were grade 1 or mild, five were grade 2 or moderate and four were grade 3 or severe), 14 specimens diagnosed with oral VC, and 15 specimens diagnosed with oral ED (six cases of mild, eight cases of moderate and four cases of severe dysplasia). The specimens were retrieved from the archives of the Oral and Maxillofacial Pathology Department of School of Dentistry, Mashhad University of Medical Sciences. The specimens were stained with hematoxylin and eosin and the slides were counted by two oral and maxillofacial pathologists to confirm accurate diagnosis and ensure the adequacy of specimens. We did not use a software analyzer for counting. Suitable areas for staining were marked. Two 4 µ-thick sections were made of each paraffin block for IHC staining. After deparaffinization and immersion in phosphate buffered saline, the sections were IHC-stained using the En-Vision method via the streptavidin-biotin interaction. Angiogenesis was quantified by assessing the expression of CD34 marker using monoclonal mouse anti-human CD34 class 2 antibody (clone QBEnol 10, ready-to-use 6 mL, code no: IS 632 30). Presence of TAMs was determined by assessment of the expression of CD68 marker using monoclonal mouse anti-human CD68 antibody (clone KP1, ready-to-use 6mL, code no: IS 60930) according to the manufacturer's instructions (Biogen company). The stained slides were evaluated by two pathologists blinded to the type of lesion under a light microscope (LABOMED, USA) at x400 magnification. Accuracy of staining was confirmed by comparing the specimens with the positive (hemangioma specimen for CD34 and tonsillar tissue for CD68 marker) and negative (specimens devoid of the primary antibody) controls. The inclusion criteria were paraffin blocks with adequate amount of tissue for IHC staining and confirmed diagnosis. Specimens with doubtful diagnosis, necrotic tissue or inadequate sample were excluded. To count the stained cells, the slides were first evaluated under a low power field, and areas with rich cellular staining were chosen. Counting was done in five areas rich in stained cells and then the mean value was calculated (8,21).

Determination of MVD

In slides stained for CD34, MVD was also determined. In five high power fields (x400 magnification) with maximum vascular density, number of cells or cell islets with brown cytoplasmic staining was counted in 1 mm 2 surface area. Vessels showing branching, cross-sectioning alongside each other, were counted as one, except for instances where discontinuity was evident in which, they were counted as two separate vessels. Separate vessels were counted separately. The mean number of vessels counted in these fields was calculated and recorded separately for each specimen (Figure 1).

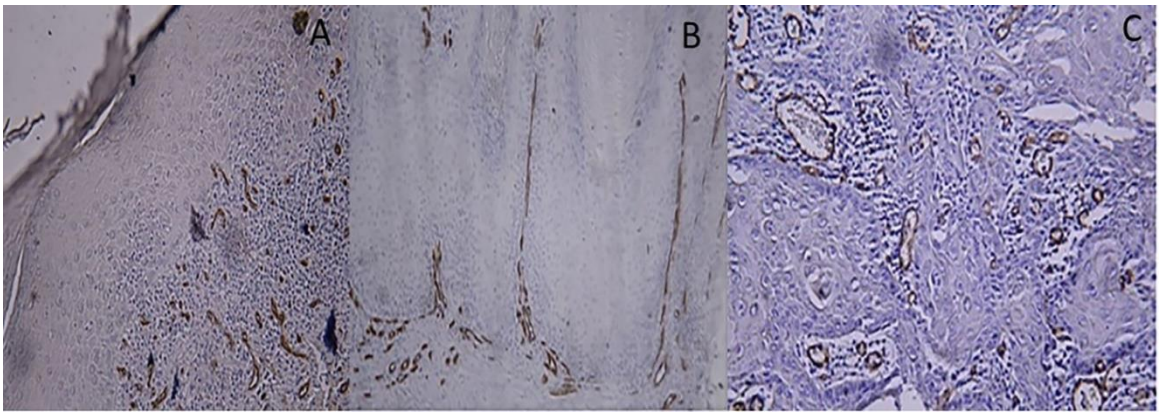

Figure 1: CD34-stained blood vessels in (A) oral epithelial dysplasia (×20), (B) oral verrucous carcinoma (×10) and (C) oral squamous cell carcinoma (×20)

Determination of the number of TAMs

In slides stained for CD68, number of TAMs showing brown cytoplasmic staining was counted in five high power fields with the highest cell density. For each specimen, the mean number of macrophages counted in these fields was calculated and separately recorded. Figure 2).

The mean and standard deviation of MVD and TAMs were calculated for each group. Data were analyzed using the Fisher's exact test, Chi-square test, Kruskal Wallis test and one-way ANOVA with the aid of SPSS version 21.

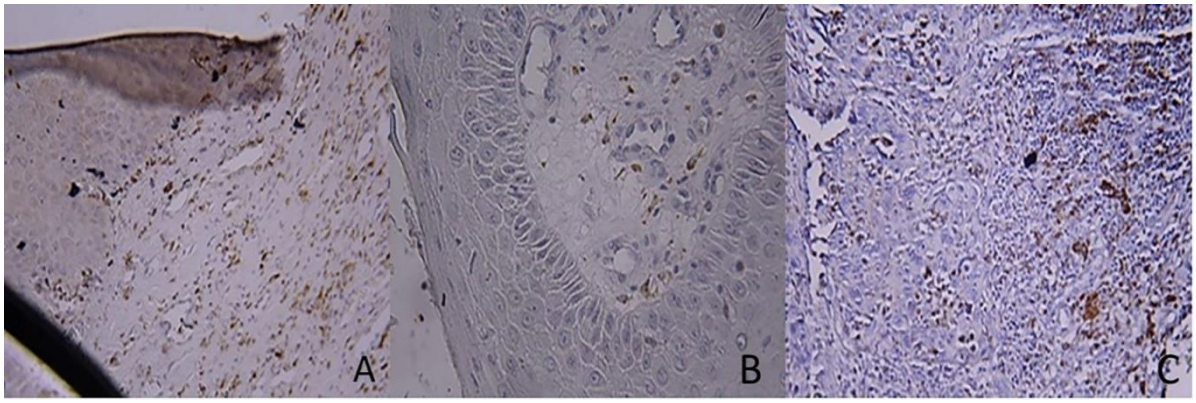

Figure 2: CD68-stained macrophages in (A) oral epithelial dysplasia (×20), (B) oral verrucous carcinoma, and (×40) (C) oral squamous cell carcinoma (×20)

Results

Data regarding oral VC, ED and SCC of 43 patients with a mean age of 59.65±17.02 years (range 23 to 84 years) and median of 62 years were analyzed (Table 1). The expression of CD68 and CD34 markers was evaluated in these specimens and compared (Table 1). One-way ANOVA analysis showed that age of patients was not significantly different in the three groups of ED, VC and SCC (P=0.145). Gender distribution was almost the same in the three groups (P=0.727). (Table 1)

Table 1. Clinical characteristics and expression of CD68 and CD34 markers in the three groups

GROUP	NO.	FEMALE (%)	MALE (%)	MEAN OF AGE ± SD	MOST FREQUENTLY LOCATED (%)	MEAN OF CD34 ± SD	MEAN OF CD68 ± SD
EPITHELIAL DYSPLASIA	15	8 (53.3%)	7 (46.7%)	53.20 ± 16.57	Tongue (46.7%)	22.47± 1.24	14.86± 18.06
VERRUCOUS CARCINOMA	14	7 (50.0%)	7 (50.0%)	65.50 ± 15.50	Vestibule (64.3%)	19.85± 1.23	9.86± 4.91
SQUAMOUS CELL CARCINOMA	14	9 (64.3%)	5 (44.2%)	60.71 ± 17.72	Tongue (57.1%)	17.56±1.25	15.64±12.11
TOTAL	43	24 (55.8%)	19 (44.2%)	59.65 ± 17.02	Tongue (37.2%)		
TEST RESULT		Chi-square X^2=0.637 P=0.727		One-way ANOVA F=2.02 P=0.145	Fisher's Exact Test P=0.004	One-way ANOVA F=2.02 P=0.145	Kruskal Wallis X2=1.98 P=0.371

A significant difference was observed among the three groups in terms of the percentage of staining for CD34 marker. Tukey's test showed that the mean expression of CD34 marker in ED was significantly higher than that in oral SCC (P=0.010). No significant difference was noted in this regard in other groups (P=0.145). The Kruskal Wallis test showed no significant difference among the three groups in terms of the percentage of staining for CD68 marker (P=0.371) (Table 1). It was revealed that the percentage of staining for CD34 and CD68 had no significant correlation with gender (P=0.707, P=0.646). The Spearman's correlation coefficient showed a very weak and insignificant correlation between age and percentage of staining for CD34 and CD68. In this regard, by an increase in age, the staining of these markers slightly decreased.

For ED, the lowest frequency was observed in the lips and buccal vestibule (n=1, 6.7%). The lowest frequency of VC was noted in the lips (n=0). For the SCC, the lowest frequency was seen in the buccal vestibule (n=0). There was a significant association between the location and the type of lesion (P=0.004) (Table 1). It was found that the mean intensity of staining for CD68 was slightly higher in severe grade compared to other grades, while the mean intensity of staining for CD34 in the severe grade was slightly lower than that in other grades. Thus, the Kruskal Wallis test was applied and revealed that the mean percentage of staining for CD34 (P=0.407) and CD68 (P=0.480) was not significantly different for various grades.

For ED, the mean intensity of staining for CD68 in low grades was slightly higher than that in other grades, while the mean intensity of staining for CD34 in severe grades was slightly higher than that in other grades. Thus, one-way ANOVA was applied and showed that the mean percentage of staining for CD34 (P=0.230) and CD68 (P=0.902) was not significantly different for various grades of ED. However, in SCC, the mean intensity of staining for CD68 marker in severe grade was higher than that in other grades, while the mean intensity of staining for CD34 in moderate grade was slightly higher than that in other grades. The mean percentage of staining for CD34 (P=0.574) and CD68 (P=0.558) was not significantly different in various grades of oral SCC (Table 2).

Table 2. Mean, standard deviation, minimum and maximum percentage of staining for CD34 and CD68 markers in different grades

Lesion/Marker/Grade			Number	Mean and standard deviation	Minimum	Maximum	P-value
Epithelial dysplasia	CD68	Low	6	17.50 ±720.6	.00	47.00	F=0.104 P=0.902
		Moderate	7	12.57 ± 18.37	.00	53.00	
		Severe	1	15.00±.	15.00	15.00	
	CD34	Low	6	25.33± 6.24	18.00	33.00	F=1.67 P=0.230
		Moderate	8	20.81± 3.64	17.00	28.50	
		Severe	1	26.00±.	26.00	26.00	
Squamous cell carcinoma	CD68	Low	5	10.80± 10.18	.00	22.00	F=0.616 P=0.558
		Moderate	5	17.40 ± 15.82	.00	40.00	
		Severe	4	19.50± 9.98	11.00	33.00	
Verrucous carcinoma	CD34	Low	5	17.55± 1.61	16.25	20.25	F=0.612 P=0.574
		Moderate	5	19.80± 6.49	12.75	27.25	
		Severe	4	16.25± 82.8	12.50	19.50	

Discussion

Considering the higher prevalence of these lesions in older individuals, this finding was expected. In the study by Shetty in contrast to our study, the highest and the lowest prevalence of carcinoma were reported in the buccal vestibule and the palate, respectively (22). This variation may be due to the different habits of individuals in different geographical regions (3). In the study by El-Rouby, positive expression of CD68 marker was observed in all specimens of SCC and VC and despite the fact that the mean percentage of CD68 expression in his study was less than that in the current study, El-Rouby's results, similar to ours, showed an insignificant increase in the expression of this marker in high-grade malignancies. The increase in expression of CD68 in different grades of cancers in El-Rouby's study was the same as that in our study (8).

In line with our findings, He et al. stated that expression of markers related to TAMs (CD68 and CD163) in oral SCC was much higher than that in ED, and the higher the grade of dysplasia and malignancy, the higher the expression of these markers would be (23). This finding indicates that TAMs play an important role in providing suitable conditions for the progression of tumors. Similarly, our study showed that CD68-positive macrophages were concentrated around the tumoral cells, which was in accord with the observations of shigeoka et al. According to Shigeoka et al, it appears that focal accumulation of immunocytes around tumors is a suitable prognostic factor for SCC (24). On the other hand, since the growth potential is greater in neoplastic lesions with a stroma containing higher frequency of blood vessels, the newly formed vessels within the tumor serve as a key factor in invasion and prognosis of tumor since they enable the tumor to metastasize.

In contrast to our study, Chawla et al. compared TAMs and angiogenesis (MVD) in ED, VC and SCC in comparison with normal oral mucosa using CD31 and CD68 antibodies and indicated that the expression of CD68 in oral SCC was higher than that in oral ED and VC. Also, they showed no association between TAMs and MVD and reported that the maximum expression of CD68 occurred in SCC, which was in agreement with our findings. Chawla et al, also evaluated angiogenesis and showed that expression of CD31 in oral SCC was higher than that in oral ED and oral VC in contrast to our study (21). High sensitivity and specificity of CD34, which is a surface antigen

in hematopoietic progenitor cells in fetal stages and in vascular endothelial cells, has been previously confirmed. Thus, expression of CD34 was evaluated in the current study (11,12). Higher MVD in ED may be due to the fact that in our study, although blood vessels had a higher frequency in ED, they were small and immature, while in VC and especially SCC, the blood vessels were mature and had a larger lumen. On the other hand, Davidson et al. evaluated cervical SCC and found no significant difference in MVD of ED and SCC, which was in line with our findings, despite the fact that they used CD31 endothelial marker (16). Thus, it may be concluded that some endothelial markers other than CD34 also play a role in the progression of tumors, and their expression increases in more severe lesions. It is important to mention that the sample size in the study by Chawla et al. was greater in comparison to our study. Assessment of the correlation of factors such as age, gender, tumor grade with the percentage of staining of markers in different lesions was among the strengths of our study compared to theirs (21).

In contrast to our study, Mallick et al. evaluated the proliferation of endothelial cells by assessing the expression of CD34 marker and MVD and reported lower expression and lower MVD in verrucous hyperplasia than in VC and SCC (25). Thus, further studies are required in this respect. In the current study, in contrast to the study conducted by Ascani et al, the highest expression of CD34 was observed in moderate-grade SCC and its expression was higher in lower grades than in higher grades. In the study by Ascani et al, severe grades of ED showed the highest expression of CD34 (6). Similarly, Shahsavari et al. concluded that angiogenic factors other than CD34 may play a fundamental role in the difference in clinical behavior of oral SCC with different grades (26). Wadhawn et al. evaluated different endothelial markers by morphometric analysis of different grades of SCC and showed that in high grade, endothelial markers such as MVD had higher expression but no significant difference was observed in low and moderate grades (27). Moreover, Kim et al. showed that the expression of VEGF and mRNA of VEGF in invasive oral SCC was much higher than that in in situ carcinoma (28).

In the study by Borase et al., expression of VEGF gene with C/T 460 polymorphism (endothelial growth gene) in oral SCC was higher than that in VC and ED (29). The above-mentioned findings confirm that CD34 may play no role in the progression of lesions from dysplasia to oral SCC or their grading from low to severe, so CD34 should be considered in combination with some other markers. Moreover, although the role of angiogenesis in oral SCC has yet to be clearly identified, recent studies have shown that non-angiogenesis mechanisms (tumor-linked vessels) may be involved in the occurrence of specific tumors (12). On the other hand, some studies using CD68, CD204 and CD163 markers for identification of TAMs showed a higher frequency along with some endothelial markers such as VEGF and TP in oral SCC with severe grade than in other groups. This indicates that TAMs and endothelial markers play an important role in the progression of malignant lesions. Thus, there is a possibility that TAMs play a role in angiogenesis in tumoral lesions; whereas, in some studies such as Chawla et al. and Dinney et al., like our study, no significant association was found between MVD and TAMs (21,30). Robbin et al also reported that MVD (determined by the expression of CD34) was not a prognostic factor for prostate cancer in males. There is a possibility that the small sample size or the method of determining MVD be responsible for such findings (31).

Our study showed that the mean expression of CD34 and CD68 was the highest in severe grade ED and high grade SCC, respectively these findings indicate that angiogenesis and TAMs play a role in the progression of lesions to tumors because the increase in expression of these markers in oral SCC was greater than that in other low-grade lesions. Our study highlighted the role of CD68 marker (related to TAMs) in the progression of lesions, but CD34 played no role in the progression of lesions to malignancy. Considering the fact that no association was found between CD34 and CD68 expression, it seems that some endothelial markers other than CD34 play a role in the progression of lesions to malignancy. Despite the high sensitivity and specificity of CD34, it cannot be used for the differentiation of normal and tumoral endothelial cells and no endothelial marker has been identified by the researchers to differentiate these two types of cells. Moreover, since tumor growth is a complex process depending on the genetic coding of tumoral cells and stromal changes around the tumor, further studies are required to determine the role of TAMs in angiogenesis.

Conclusion

Considering the controversial results regarding the association of MVD and TAMs and the significance of early diagnosis of premalignant and malignant lesions, further complementary studies with a larger sample size are required to assess the efficacy of other IHC markers for this purpose.

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